

Mycotoxin from a Blue-Eye Mold of Corn¹

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High-moisture yellow dent corn became heavily molded by *Penicillium martensii* after storage for 6 months at 1 C. Mice ingesting corn molded by *P. martensii* died within a few days. The toxin was isolated and identified as penicillic acid. Large quantities of the toxin accumulated over a 3-month period on artificially inoculated corn incubated at temperatures between 1 and 15 C. At higher temperatures, the toxin disappeared within 45 days.

Recent changes in agricultural technology have resulted in the widespread practice of harvesting high-moisture corn by picker-sheller. The corn frequently has a moisture level in excess of 20% and has sustained considerable physical damage. Such conditions are ideal for rapid molding. After harvesting, the corn must either be dried to a moisture level safe for storage or be ensiled and used for animal feed. Several commercial firms in this country and abroad have considered refrigerated storage of high-moisture corn to be an attractive alternative to drying or ensiling (6). This method of preservation allows the corn to be used for feed, or it can be channeled to the milling industry. Even under refrigeration, however, corn is known to mold, and guidelines for cold storage have been proposed (13).

During storage of high-moisture yellow dent corn for 6 months at 1 C, the mold flora shifted, and *Penicillium martensii* Biourge predominated. This paper reports the isolation and identification of a mycotoxin from corn molded by *P. martensii* NRRL 3612 and the temperatures at which toxin production occurs.

MATERIALS AND METHODS

Source of corn from which *P. martensii* was isolated. This corn was a commercial single-cross yellow dent grown on the Agricultural Engineering farm, University of Illinois, Urbana. The corn was planted on 5 June and harvested by picker-sheller on 29 October 1968. The moisture level at harvest was 25% and was determined electrically and by oven drying.

Microbiological examination of corn. Fifty-gram samples of corn were shaken vigorously in 450 ml of sterile distilled water containing 25 g of sand, and serial dilutions were then made. Bacterial counts were

determined on plate count agar containing 30 μ g of Acti-Dione per ml (Upjohn; cycloheximide) and mold counts on yeast extract-tryptone agar (5) containing 100 μ g of Achromycin per ml (Lederle; tetracycline-KCl). Bacterial colonies were counted after incubation for 3 days at 32 C; molds and yeasts, after incubation for 5 days at 28 C.

Animal toxicity tests. Molded corn was extracted as described below, and, after solvent removal, the residue was dissolved in propylene glycol. The extracts were tested on mice by intraperitoneal injection.

Fermentation and toxin isolation. Sizable quantities of the toxin were obtained from a liquid medium. Fifteen liters of Czapek-Dox broth (9) supplemented with 0.5% yeast extract was distributed into 30 Fernbach flasks (2.8 liter), autoclaved, inoculated with an aqueous spore suspension of *P. martensii*, and incubated statically for 12 days at 25 C. The mycelium was removed by filtration, and the supernatant was concentrated to 0.5 liter in a vacuum evaporator. The concentrated supernatant was extracted twice by using 1 liter of chloroform for each extraction. The chloroform extracts were combined, and most of the solvent was removed by flash evaporation. After the residual oily liquid was added to 20 volumes of pentane-hexane, the precipitate was recovered by filtration and then redissolved in boiling water. On cooling, white crystals precipitated. These were recovered, air-dried, and recrystallized from benzene twice, as fine needle-shaped crystals (total recovery about 130 mg). Later, considerably better yields were obtained by the use of Raulin-Thom medium (4) in place of Czapek-Dox supplemented with yeast extract.

Toxin identification. The molecular weight and elemental formula of the toxin were determined with a Euclid high-resolution mass spectrometer. Melting points were obtained with a Mettler FPI melting point apparatus, and excitation and emission spectra were recorded with an Aminco-Bowman spectrophotofluorometer. Ultraviolet absorption spectra were determined with a Beckman DB-G spectrophotometer.

Production of toxin on corn. Fifty-gram quantities of corn were placed in 300-ml Erlenmeyer flasks with 100 ml of distilled water and autoclaved for 15 min at 121

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C. After autoclaving, the excess water was decanted, and the flasks, stoppered with cotton plugs, were then autoclaved for 20 min. Each flask was inoculated with 1 ml of spore suspension made by suspending the spores of *P. martensii* from a 10-day-old slant in 50 ml sterile distilled water. The flasks were incubated at -4, 1, 5, 10, 15, 20, 25, 30, 32, 35, and 37 C.

Isolation and assay of toxin. The toxin, penicillic acid, was assayed fluorodensitometrically by the method of Ciegler and Kurtzman (*in preparation*). Briefly, the method involves thin-layer chromatography of the unknown with known amounts of standard on silica gel (solvent, chloroform-ethyl acetate-formic acid, 60:40:1, v/v) followed by exposure of the plate to concentrated ammonia. Penicillic acid is excited at 350 nm and fluoresces at 440 nm. The degree of fluorescence was determined with a Photovolt model 530 densitometer equipped with an automatic-scanning thin-layer plate stage and a recorder equipped with an integrator. A standard curve is prepared for each analysis, Beer's law being followed between 1 and 9 μ g of penicillic acid. The concentration of unknown is determined from the standard curve, taking into account the dilutions involved.

For most assays, 50 g of molded corn was extracted with 250 ml of chloroform-methanol (90:10, v/v) in a Waring Blendor for 3 min. The first 50 ml of solvent, after recovery by filtration through anhydrous sodium sulfate, was analyzed for penicillic acid.

TABLE 1. *Microflora of high-moisture yellow dent corn at harvest and after storage at 1 C for 6 months*^a

Microorganisms	Before storage	After storage
	no./g	no./g
Bacteria.....	242,000	248,000
Yeasts.....	10,000	8,000
Mucoraceous fungi.....	1,000	200
<i>Fusarium</i> spp.....	114,000	900,000
<i>Penicillium</i> spp. (other than <i>Penicillium martensii</i>).....	7,000	0
<i>P. martensii</i>	1,000	1,800,000

^a Moisture level: 25%.

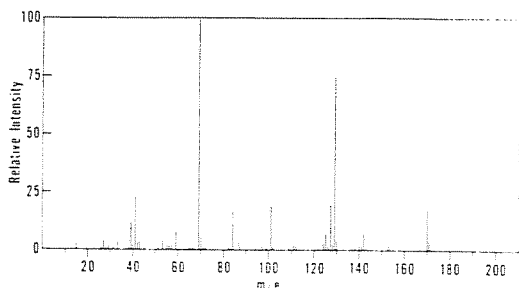


FIG. 1. Mass spectrum of penicillic acid from *Penicillium martensii*. Spectra of authentic penicillic acid and that from *P. martensii* are superimposable.

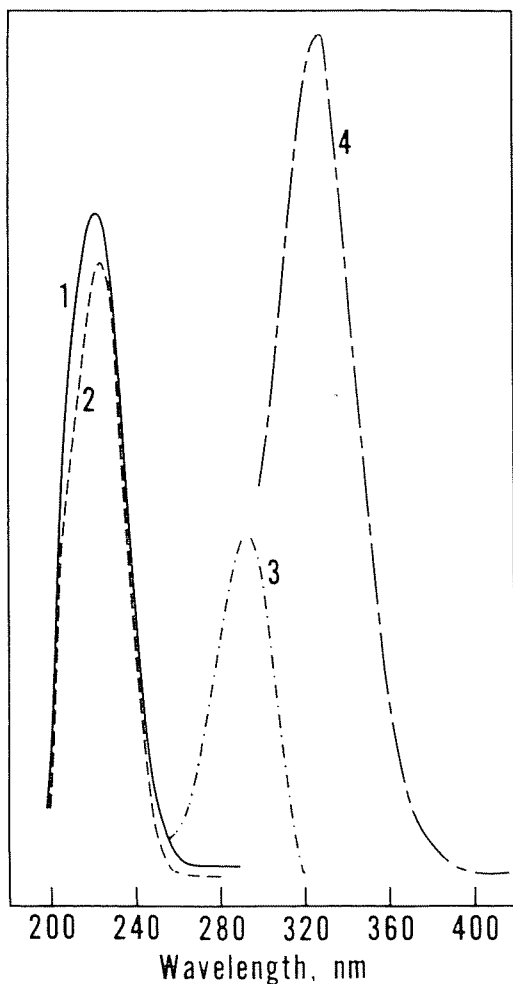


FIG. 2. Ultraviolet absorption spectra of penicillic acid: (1) in methanol, (2) in 0.02 N HCl, (3) in 0.02 N NaOH, and (4) derivative formed by reaction of penicillic acid with ammonia. Spectrum recorded in methanol.

RESULTS

Yellow dent corn with a moisture level of 25% was stored for 6 months at 1 C in sealed screw-cap 1-quart (ca. 1.1 liters) glass jars. Before storage, the penicillia numbered about 8,000/g, but after storage the count increased to 1,800,000/g (Table 1). Although several species of *Penicillium* were present before storage, only one species, *P. martensii*, was recovered after storage. Initially, *P. martensii* caused blue-eye of the kernels but eventually spread over the surface of the entire grain.

Intraperitoneal injections of extracts of corn molded by *P. martensii* were fatal to mice in a

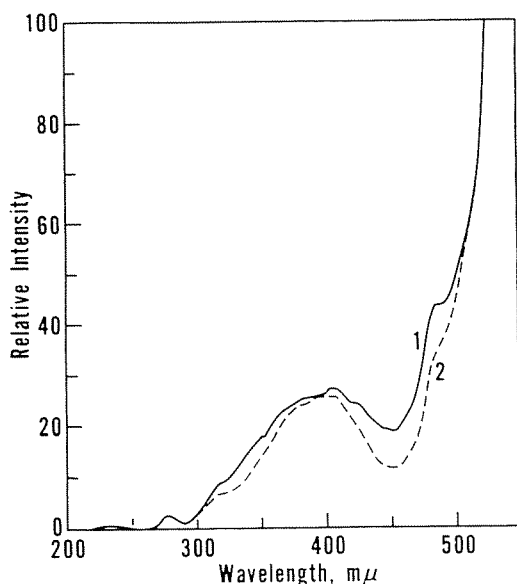


FIG. 3. Excitation spectrum of penicillic acid hydrazone. Spectrum recorded in methanol with spectrofluorometer. (1) Penicillic acid from *P. martensii*. (2) Authentic penicillic acid.

matter of minutes. Mice fed the molded corn died in 3 to 5 days.

The toxin was identified as penicillic acid by analyzing both authentic penicillic acid and the isolated product. High-resolution mass spectroscopy gave m/e 170.06 and an elemental formula of $C_8H_{10}O_4$ (Fig. 1). The melting point was 84.2 to 84.8 C with no depression on admixture with authentic penicillic acid. The ultraviolet absorbance in methanol showed a single

peak at 221 nm with shifts to 224 nm on acidification with 0.02 N HCl and to 293 nm in 0.02 N NaOH (Fig. 2). After reaction with ammonium hydroxide, the keto form of the acid fluoresces with excitation at 350 nm and emission at 440 nm. The toxin was co-chromatographed with authentic penicillic acid in several solvent systems and both compounds, after reaction with phenylhydrazine in ammonia, gave identical excitation spectra (Fig. 3).

Production of penicillic acid on corn by *P. martensii* was favored by low temperatures. Greatest production (12.7 mg/g) was at 5 C after 88 days, but nearly two-thirds of this amount occurred at 1 and 10 C (Table 2). After 83 days, penicillic acid was also detected at -4 C. Considerable synthesis occurred at 15 and 20 C, but the toxin disappeared after 45 and 90 days, respectively. Only small amounts of toxin were detected at 30 and 32 C, and there was no growth or toxin production at 35 C.

DISCUSSION

Blue-eye is a storage disease of corn caused by several species of *Penicillium*. These molds grow over the embryo but under the seed coat. When sporulation occurs, a blue or blue-green color appears over the embryo because of the color of the spores. Koehler (7) reported *P. notatum* Westling, *P. viridicatum* Westling, *P. palitans* Westling, and *P. cyclopium* Westling to cause blue-eye; Semeniuk and Gilman (11) list *P. expansum* Link; and Semeniuk (10) added *P. rugulosum* Thom and *P. chrysogenum* Thom. Our report is the first of blue-eye being caused by *P. martensii*. However, the similarity of *P.*

TABLE 2. Effect of time and temperature on the production of penicillic acid on corn by *P. martensii*

Temp	Amt (mg/g of dry corn) of penicillic acid produced in various time periods												
	5 days	12 days	14 days	16 days	21 days	26 days	45 days	60 days	79 days	83 days	88 days	117 days	128 days
C													
-4	— ^a	—	—	—	—	—	—	—	—	0.22	—	1.30	2.42
1	—	—	ND ^b	—	ND	0.34	2.40	3.28	5.60	7.50	—	—	8.20
5	—	—	ND	—	0.43	2.80	5.10	5.75	—	—	12.70	—	7.70
10	—	—	1.58	—	3.66	5.00	7.35	6.60	—	7.50	—	—	—
15	0.36	4.43	—	4.91	5.85	5.30	6.20	—	—	0.13	—	—	—
20	1.92	5.78	—	4.31	3.88	4.30	—	—	—	—	—	—	—
25	1.60	3.01	—	1.60	1.51	1.70	ND	—	—	—	—	—	—
30	0.20	0.48	—	0.26	0.09	0.20	ND	—	—	—	—	—	—
32	0.28	0.52	—	0.32	ND	—	—	—	—	—	—	—	—
35	NG ^c	NG	—	NG	NG	—	—	—	—	—	—	—	—

^a No assay.

^b ND, no penicillic acid detected.

^c NG, no detectable growth.

martensii and *P. cyclopium* may have resulted in it being confused with the latter.

Penicillic acid was first isolated by Alsberg and Black (1) from *P. puberulum* Bainier. This culture, isolated from corn, produced sufficient penicillic acid on Raulin's medium to be fatal to mice and guinea pigs. Murnaghan (8) considerably expanded this early work in his studies of the pharmacology of penicillic acid. The intravenous LD₅₀ of mice was 5 mg/20 g, and the mean lethal dose when given orally was 12 mg/20 g. Penicillic acid had a digitalis-like action on the heart of the frog, the rabbit auricle, the perfused heart of the cat, and a very weak action in heart-lung preparations of the dog. A dilator action on systemic blood vessels was also found and included the coronary and pulmonary vessels.

In our investigation, the greatest accumulation of penicillic acid on corn inoculated with *P. martensii* occurred at temperatures of 10 C and below. Production at 15 and 20 C was also high, but the toxin disappeared within 45 and 90 days, respectively. Temperatures above 25 C were decidedly unfavorable for production. Several interpretations can be made of these data. Degradation of toxin at the higher temperatures may result from a nonspecific autocatalytic process or may be enzymatic; at lower temperatures, the rate of production may exceed that of degradation, or the degradation process, whether nonspecific or enzymatic, may not function. This facet of our data requires additional experimentation.

Mycotoxins produced on grains at low temperatures have previously been reported and became a serious problem in the Soviet Union in the early 1930's (3). A human disease now known as alimentary toxic aleukia (ATA) became widespread as a result of the consumption of moldy grain which had overwintered in the field. Various species of *Fusarium* and *Cladosporium* were apparently responsible for ATA, with greatest toxin production occurring at -10 to 0 C. Similarly, greatest production of T-2 toxin by *Fusarium tricinctum* (Corda) Sacc. emend. Snyder and Hansen, which has been associated

with moldy corn toxicoses, occurred at low temperatures (2).

The importance of penicillic acid as a mycotoxin on high-moisture corn stored at low temperatures must be further assessed by additional field studies and animal feeding trials. Furthermore, toxin production may not be limited to *P. martensii*. Other fungi producing blue-eye, such as *P. chrysogenum*, *P. palitans*, and *P. rugulosum*, have been shown to grow at 0.5 C (12). Their toxin-producing ability at various temperatures is currently under study in our laboratory.

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